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Tatsuhiko KODAMA, et al.

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FOR: LKLF/KLF2 GENE EXPRESSION PROMOTER

CONFIRMATION OF FILING AN ENGLISH TRANSLATION AND STATEMENT IN PRIOR FILED PROVISIONAL APPLICATION

COMMISSIONER FOR PATENTS
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Sir:

The undersigned hereby confirms the filing of an English translation and statement that the translation is accurate in prior-filed Application Number 60/463,311.

Respectfully Submitted,

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DECLARATION

I, Yuko Onoda of c/o The Patent Corporate Body ARUGA PATENT OFFICE, 3-6, Nihonbashiningyocho 1-chome, Chuo-ku, Tokyo 103-0013 Japan do solemnly and sincerely declare that I well understand both Japanese and English languages and that I believe the attached English version is a true and complete translation of the extract of the U.S. patent provisional application No. 60/463,311.

June 22, 2007

Yuko ONODA

DESCRIPTION

LKLF/KLF2 GENE EXPRESSION PROMOTING METHOD

Technical Field

This invention relates to lung Kruppel-like factor/KLF2 (hereinafter abbreviated as "LKLF/KLF2") gene expression promoting method, which are useful for the treatment and/or decrudescence of diseases associated with blood vessels, for example, diabetes, effort angina, unstable angina, angina pectoris decubitus, myocardial infarction, atherosclerosis, hemoendothelial function disorder, post-PTCA restenosis, hypertensivity pneumonitis, interstitial pneumonia, airway constriction, airway obstruction, eyeground bleeding (retinal vein occlusion, vitreous floaters, etc.), cerebrovascular dementia, cerebral infarction, cerebral hemorrhage, subarachnoid hemorrhage, hemorrhoid, and the like.

Background Art

LKLF/KLF2 is a transcription regulatory factor protein having structures of proline-rich repeats, an activation domain, a nuclear localization signal and a zinc finger domain [Kozyrev S.V., et al., FEBS Lett., 448(1), 149-52, April 1, 1999]. As an effect of LKLF/KLF2, LKLF/KLF2 is known *inter*

alia to be important for hemocyte differentiation [Kuo C.T., et al., Genes Dev., 11(22), 2996-3006, 1997; Anderson K.P. et al., Mol. Cell Biol., 15(11), 5957-65, 1995], to be an important signal transduction factor between vascular endothelial cells and smooth muscle cells [Monajemi H., et al., Thromb. Haemost., 86(1), 404-12, 2001], to decrease proliferation of T cells, to reduce cell size and protein synthesis and to decrease surface expression of activation markers [Buckley A.F., et al., Nat. Immunol., 2(8), 698-704, 2001], and further, to be essential for blood vessel stabilization [Kuo C.T., et al., Genes Dev., 11(22), 2996-3006, 1997].

On the other hand, initial lesions of atherosclerosis are known to frequently occur at vessel bifurcations and curvatures where blood flow varies significantly. As a cause of their occurrence, shear stress of the blood flow on the vascular endothelium is considered to play a role. According to recent reports, however, it is pointed out that LKLF/KLF2 is expressed from vascular endothelial cells under shear stress [Dekker R.J., et al., Blood, 100(5), 1689-98, 2002] and that LKLF/KLF2 suppressively takes part in the occurrence of atherosclerosis [Karin Arkenbout E., et al., Thromb. Haemost., 89(3), 522-9, 2003]. As appreciated from the foregoing, LKLF/KLF2 which is expressed from vascular endothelial cells is presumed to suppressively act on lesions associated with

blood vessels.

Promotion of LKLF/KLF2 gene expression is expected to achieve decrudescence or treatment of diseases associated with blood vessels which is led by arterial sclerosis etc. However, no substance which is able to promote the expression of LKLF/KLF2 gene, including physiological ones, has been known so far.

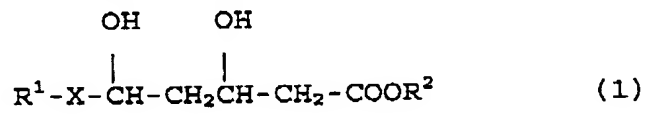
An object of the present invention is therefore to provide an LKLF/KLF2 gene expression promoting method effective for the treatment and/or decrudescence of diseases associated with blood vessels, for example, diabetes, effort angina, unstable angina, angina pectoris decubitus, myocardial infarction, atherosclerosis, hemoendothelial function disorder, post-PTCA restenosis, hypertensivity pneumonitis, interstitial pneumonia, airway constriction, airway obstruction, eyeground bleeding (retinal vein occlusion, vitreous floaters, etc.), cerebrovascular dementia, cerebral infarction, cerebral hemorrhage, subarachnoid hemorrhage, hemorrhoid, and the like.

Disclosure of the Invention

Using a cultured human cell system, the present inventors have searched for substances which might affect the expression of LKLF/KLF2 gene. As a result, it has been found that compounds represented by the below-described formula (1) which are known

as HMG-CoA reductase inhibitors and their lactone derivatives and salts of the compounds and derivatives, especially pitavastatin calcium has activities to promote the expression of LKLF/KLF2 gene, leading to the completion of the present invention.

The present invention provides an LKLF/KLF2 gene expression promoting method, which comprises administering, as an active ingredient, an effective amount of a compound, which is represented by the following formula (1):



$$R^1\text{-}X\text{-}CH(OH)\text{-}CH_2CH(OH)\text{-}CH_2\text{-}COOR^2 \qquad (1)$$

wherein R^1 represents an organic group, X represents $-CH_2CH_2-$ or $-CH=CH-$, and R^2 represents a hydrogen atom or an alkyl group, or a lactone derivative thereof, or a salt thereof.

The present invention provides an LKLF/KLF2 gene expression promoter, which comprises as an active ingredient a compound represented by the formula (1), or a lacton derivative thereof, or a salt thereof.

The present invention further provides use of a compound, which is represented by the formula (1), or a lactone derivative thereof, or a salt thereof as an active ingredient for the production of an LKLF/KLF2 gene expression promoter.

Brief Description of the Drawings

FIG. 1 is a diagram showing expression levels of

LKLF/KLF2 gene in the presence of pitavastatin calcium.

Best Modes for Carrying Out the Invention

Compounds represented by the formula (1), their lactone derivatives and salts of these compounds and lactone derivatives, all of which are usable in the present invention, are known as HMG-CoA reductase inhibitors useful as hyperlipidemia therapeutics, but it has not known whether these compounds affect gene expression of LKLF/KLF2 or not.

The organic group represented by R^1 in the compound represented by the formula (1) may preferably be a substituted or unsubstituted organic group having a cyclic structure.

Examples of the organic group having the cyclic structure include indolyl, indenyl, pyridyl, pyrrolopyridyl, pyrazolopyridyl, thienopyridyl, pyrimidyl, pyrazolyl, pyrrolyl, imidazolyl, indolidyl, quinolyl, naphthyl, hexahydronaphthyl, cyclohexyl, phenylsilylphenyl, phenylthienyl and phenylfuryl groups, with hexahydronaphthyl, indolyl, pyridyl, pyrimidyl, pyrrolyl and quinolyl groups being particularly preferred.

Examples of substituent groups, which may substitute on these organic groups having the cyclic structures, include hydroxyl group, linear, branched or cyclic alkyl groups, alkyloxyalkyl groups, alkylcarbonyloxy groups, alkyl-substituted amino groups, substituted

alkylsulfonylamino groups, substituted phenylsulfonylamino groups, carbamoyl group which may be substituted by one or two alkyl or phenyl groups, halophenyl groups, alkylphenyl groups, alkoxyphenyl groups, phenyl group, and oxo group.

Among these substituents which may substitute on these organic groups having the cyclic structures, preferred are linear, branched or cyclic C_{1-6} alkyl groups, C_{2-7} alkyloxyalkyl groups, C_{1-4} acyloxy groups, C_{1-4} alkyl-substituted amino groups, C_{1-4} alkyl-substituted C_{1-4} alkylsulfonylamino groups, C_{1-4} alkyl-substituted phenylsulfonylamino groups, C_{1-4} alkyl-substituted carbamoyl groups, phenyl-substituted carbamoyl groups, fluorophenyl groups, bromophenyl groups, iodophenyl groups, methylphenyl groups, ethylphenyl groups, methoxyphenyl groups, ethoxyphenyl groups and phenyl group, with isopropyl, cyclopropyl and p-fluorophenyl groups being particularly preferred.

The lactone derivative can be obtained by subjecting its corresponding compound, which is represented by the formula (1), to lactonization in a manner known *per se* in the art, for example, under acidic conditions.

The salts of the compound represented by the formula (1) and its lactone derivative are physiologically acceptable salts. Examples thereof include alkali metal salts such as the sodium salts and potassium salts, alkaline earth metal salts such as the calcium salts and magnesium salts, organic

amine salts such as the phenethylamine salts, and the ammonium salts, with the sodium salts and calcium salts being more preferred.

These compounds are disclosed, for example, in US-A-4,739,073 and EP-A-114,027; EP-A-367,895; US-A-5,001,255, US-A-4,613,610, US-A-4,851,427, US-A-4,755,606, US-A-4,808,607, US-A-4,751,235, US-A,4,939,159, US-A-4,822,799, US-A-4,804,679, US-A-4,876,280, US-A-4,829,081, US-A-4,927,851, US-A-4,588,715; F.G. Kathawala, Medical Research Reviews, 11, 121-146 (1991), EP-A-304,063; EP-A-330,057; US-A-5,026,708, US-A-4,868,185; EP-A-324,347; EP-A-300,278; US-A-5,013,749, US-A-5,872,130, US-A-5,856,336, US-A-4,231,938, US-A-4,444,784, US-A-4,346,227, US-A-5,354,772, US-A-5,273,995, US-A-5,177,080, US-A-3,983,140, JP-B-2,648,897, US-A-5,260,440, Bioorganic & Medicinal Chemistry, 5, 437 (1977), JP-B-2,569,746, EP-B-304,063, and US-A-5,856,336.

Preferred examples of the active ingredient in the method according to the present invention for the promotion of expression of LKLF/KLF2 gene include lovastatin (US-A-4,231,938: (+)-(1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthyl (S)-2-methylbutyrate), simvastatin

(US-A-4,444,784:
(+)-(1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthyl 2,2-dimethylbutanoate), pravastatin (US-A-4,346,227:
(+)-(3R,5R)-3,5-dihydroxy-7-[(1S,2S,6S,8S,8aR)-6-hydroxy-2-methyl-8-[(S)-2-methylbutyryloxy]-1,2,6,7,8,8a-hexahydro-1-naphthyl]heptanoic acid), fluvastatin (US-A-5,354,772:
(3RS,5SR,6E)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1H-indol-2-yl]-3,5-dihydroxy-6-heptenoic acid), atorvastatin (US-A-5,273,995:
(3R,5R)-7-[2-(4-fluorophenyl)-5-isopropyl-3-phenyl-4-phenylcarbamonyl-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid), cerivastatin (US-A-5,177,080:
(3R,5S)-erythro-(E)-7-[4-(4-fluorophenyl)-2,6-diisopropyl-5-methoxymethyl-pyridin-3-yl]-3,5-dihydroxy-6-heptenoic acid), mevastatin (US-A-3,983,140:
(+)-(1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-7-methyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthyl(S)-2-methylbutyrate), rosuvastatin (US-A-5,260,440, JP-B-2,648,897:
7-[4-(4-fluorophenyl)-6-isopropyl-2-(N-methyl-N-methanesulfonylaminopyrimidin)-5-yl]-(3R,5S)-dihydroxy-(E)-6-heptenoic acid), and pitavastatin (US-A-5,856,336, JP-B-2,569,746:

(3R,5S,6E)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoic acid, and their salts. In particular, pitavastatin and its salts are preferred.

The compound represented by formula (1), its lactone derivative, the salt of the compound or lactone derivative significantly promotes the expression of mRNA of LKLF/KLF2 in human cells, and therefore, is useful in the method of the present invention for promoting the expression of LKLF/KLF2 gene and is useful for the treatment of diseases in the onset of which LKLF/KLF2 takes part, especially the treatment of diseases associated with blood vessels. Further, use of the compound represented by the formula (1), its lactone derivative, the salt of the compound or lactone derivative makes it possible *inter alia* to develop experiment systems in which LKLF/KLF2 takes part and to screen novel medicines.

Illustrative administration routes for the compound represented by the formula (1) or its lactone derivative or the salt of the compound or lactone derivative include oral administrations by tablets, capsules, a granule, a powder, a syrup and the like; and parenteral administrations by an intravenous injection, an intramuscular injection, suppositories, an inhalant, a transdermal preparation, an eye drop, a nasal drop and the like.

To formulate medicinal preparations in such various forms as described above, the active ingredient can be used

either singly or in combination with one or more of pharmaceutically acceptable excipients, binders, extenders, disintegrants, surfactants, lubricants, dispersants, buffering agents, preservatives, corrigents, perfumes, coating materials, carriers, diluents and the like, as needed.

Of these administration routes, oral administrations are preferred. Upon formulation of a medicinal preparation for oral administration, it is preferred to adjust the pH in view of the stability of the active ingredient (JP-A-2-0006406, JP-B-2,774,037, WO-A-97/23200, etc.).

The dosage of the active ingredient varies *inter* alia depending on the weight, age, sex, conditions and the like of each patient. In the case of an adult, however, it is generally preferred to orally or parenterally administer the active ingredient at a daily dosage of from 0.01 to 1,000 mg, specifically from 0.1 to 100 mg in terms of the compound represented by formula (1) at once or in several portions.

Examples

The present invention will hereinafter be described in detail based on Examples. It should however be borne in mind that the present invention is not limited to the following Examples.

Example 1

Two days after inoculation of normal human umbilical

vein endothelial cells (HUVEC) at 3 x 10^5 cells/10 cm dish, pitavastatin calcium was added to 1.1 μmol/L. Dimethyl sulfoxide, a solvent for pitavastatin calcium, was added to a control (final concentration: 0.0066 v/v%). Eight hours after the addition, total RNAs were extracted with "ISOGEN" (trade mark, product of NIPPON GENE CO., LTD.). The following procedures were conducted in accordance with the procedures manual of Affymetrix. Inc. Described specifically, following the methods known *per se* in the art, mRNA was isolated from each total RNA obtained above, and based on the mRNA, cDNA was synthesized. Further, biotin-labeled cRNA was synthesized by *in vitro* transcription. Subsequent to purification, the biotin-labeled cRNA was subjected to fragmentation by heat treatment to prepare fragmented cRNA for use in a gene expression analysis.

Gene expression analysis method: The fragmented cRNA was poured into "Human Genome Focus Array" (trade name, product of Affymetrix, Inc.), and hybridization was conducted at 45°C for 16 hours. Subsequent to washing, staining with phycoerythrin-labeled streptavidin and biotinylated antistreptavidin antibody was applied, and gene expression information was inputted by "GeneChip™ Scanner" (trade name, manufactured by Hewlett Packard Company). The information was analyzed by "GENECHIP SOFTWARE" (trade name, product of Affymetrix, Inc.) to effect a comparison in expression level.

The results of the measurements are shown in FIG. 1.

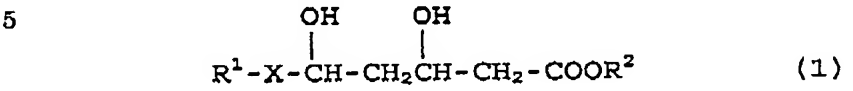
The expression of LKLF/KLF2 gene in HUVEC upon elapsed time of 8 hours after the addition of the active ingredient was significantly promoted to 761 in the pitavastatin calcium addition group as opposed to 271 in the control. Further, this effect available from the addition of pitavastatin calcium decreased to 355 by the addition of 10 μmol geranylgeranyl pyrophosphate (GGPP). Involvement of the Rho factor family in the acting mechanism of pitavastatin calcium was suggested accordingly.

Industrial Applicapability

According to the present invention, it is possible to provide a method for promoting expression of LKLF/KLF2 gene. This method is effective for the treatment and/or decrudescence of diseases associated with blood vessels, for example, diabetes, effort angina, unstable angina, angina pectoris decubitus, myocardial infarction, atherosclerosis, hemoendothelial function disorder, post-PTCA restenosis, hypertensivity pneumonitis, interstitial pneumonia, airway constriction, airway obstruction, eyeground bleeding (retinal vein occlusion, vitreous floaters, etc.), cerebrovascular dementia, cerebral infarction, cerebral hemorrhage, subarachnoid hemorrhage, hemorrhoid, and the like.

CLAIMS

1. A method for promoting expression of LKLF/KLF2 gene, which comprises administering an effective amount of a compound represented by the following formula (1):



(1)

wherein R^1 represents an organic group, X represents $-CH_2CH_2-$ or $-CH=CH-$, and R^2 represents a hydrogen atom or an alkyl group, or a lactone derivative thereof, or a salt thereof, as an active ingredient.

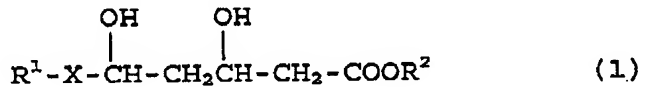
2. The method of claim 1, wherein R^1 is a substituted or unsubstituted indolyl, indenyl, pyridyl, pyrrolopyridyl, pyrazolopyridyl, thienopyridyl, pyrimidyl, pyrazolyl, pyrrolyl, imidazolyl, indolidyl, quinolyl, naphthyl, hexahydronaphthyl, cyclohexyl, phenylsilylphenyl, phenylthienyl or phenylfuryl group.

3. The method of claim 1, wherein said active ingredient is lovastatin, pravastatin, simvastatin, fluvastatin, cerivastatin, atorvastatin, rosuvastatin, mevastatin or pitavastatin, or a salt thereof.

4. The method of claim 1, wherein said active ingredient is pitavastatin or salt thereof.

ABSTRACT

The present invention provides a method for promoting expression of LKLF/KLF2 gene, which comprises administering an effective amount of a compound represented by the following formula (1):



(1)

wherein R^1 represents an organic group, X represents $-CH_2CH_2-$ or $-CH=CH-$, and R^2 represents a hydrogen atom or an alkyl group, or a lactone derivative thereof, or a salt thereof.

Fig. 1